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Perspective

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A History of the Molecular Initiating Event

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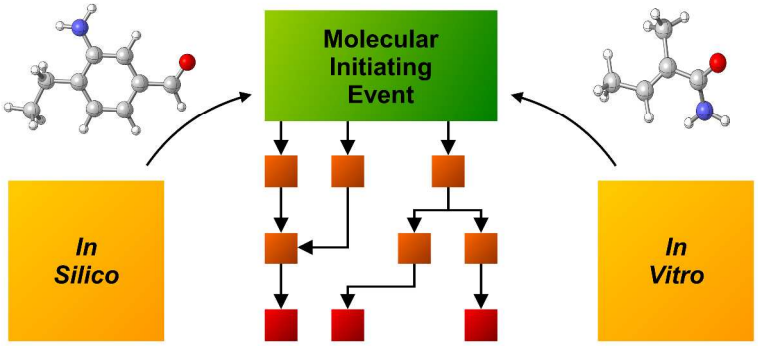

ABSTRACT

The adverse outcome pathway (AOP) framework provides an alternative to traditional *in vivo* experiments for the risk assessment of chemicals. AOPs consist of a number of key events (KEs) linked by key event relationships (KERs) across a range of biological organization backed by scientific evidence. The first KE in the pathway is the molecular initiating event (MIE); the initial chemical trigger that starts an AOP. Over the last three years the AOP conceptual framework has gained a large amount of momentum in toxicology as an alternative to animal methods and so the MIE has come into the spotlight. What is an MIE? How can MIEs be measured or predicted? What research is currently contributing to our understanding of MIEs? With this review we outline answers to these key questions.

INTRODUCTION

Toxicology risk assessment is undergoing a paradigm shift away from *in vivo* data and towards risk assessment frameworks that incorporate non-animal alternatives. Traditional *in vivo* toxicity studies form the basis of the majority of regulations globally that relate to assuring human and environmental safety. Increasingly however, there are societal, scientific and regulatory drivers to develop new ways of assuring safety that do not rely on data generation in animals. Rather than relying on apical toxicity endpoints in animals, new frameworks rely on an understanding of the mechanism of a chemical's toxicity in a relevant system which is thought to provide a more scientifically sound methodology on which to base risk assessment decisions.

The National Research Council of the United States (NRC) opened the field for discussion in 2007, in an attempt to make toxicity testing quicker, less expensive, and more relevant to human exposures.[1] It pointed to a number of advances in the fields of biology and biotechnology that make it possible to feed data into new risk assessment approaches. These include physiologically based pharmacokinetic (PBPK) modelling methods, which provide a better understanding of how a compound behaves inside the body, and how much of it is able to get to a site of action.[2,3] Furthermore, additional understanding of biological processes and the holistic nature of biology are being gained through -omics technologies[4–6] and systems biology.[7,8] *In silico* methods also have their part to play in this paradigm shift. Informatics approaches such as (Quantitative) Structure Activity Relationships ((Q)SARs) and Read-Across have the potential to make better use of existing data and target required testing to bring down the volume of *in vivo* studies required for a risk assessment.[9,10] In addition to these, the coordination of the international scientific community, including collaboration between industry, academia and regulators, is of great importance for the advance of toxicity testing.[11,12] These combined changes move toxicology away from a predominantly observational craft towards a science based on understanding.[13]

The adverse outcome pathway (AOP) framework for risk assessment is one approach to combine *in silico*, *in vitro* and *in chemico* methods with existing data to offer an alternative to animal experiments. Since it was first described in 2010, this framework has gained a lot of attention within the toxicology community. The Molecular Initiating Event (MIE) is the initial chemical-biological interaction that starts the AOP. Understanding, characterizing and predicting MIEs will be of great importance to allow the AOP framework to realize its potential as a predictive tool and this key interaction is the focus of this work. With this review we aim to cover key MIE

research between the description of the AOP framework in 2010 and the end of 2015, giving an overview of how this concept has risen to prominence, and how it will aid toxicity risk assessment of the future.

This review is focused on the MIE within human health risk assessment, but MIEs are also relevant to ecotoxicology. Many of the ideas presented are equally applicable to ecotoxicology and human health, as the MIE occurs early in the AOP and can be common to a number of species.

Up to 2012

The first mention of a Molecular Initiating Event can be traced back to 2006. Schultz *et al* identified plausible MIEs based on the covalent interaction of soft electrophiles and biological molecules.[14] These interactions can lead to a number of toxicological endpoints including skin sensitization, DNA damage and immunological responses. These MIEs are considered appropriate targets for (Q)SAR modeling, and some models are presented linking molecular reactivity at a thiol moiety to aquatic toxicity and respiratory irritation endpoints. These reactivity-driven MIEs and their subsequent toxicity pathways were a key driver for the development of the AOP framework.

The modelling of chemical interactions between electrophiles and biological macromolecules was already established before the MIE. Work by Aptula and Roberts defined mechanistic domains for reactive aquatic toxicants,[15] and the same mechanistic domains were later applied to skin sensitizers.[16] This work due to a similarity in the MIE for these toxicological endpoints, the covalent modification of proteins. This MIE can be broken down based on the type of electrophilic chemistry that causes it. These categories were used as a basis for qualitative

mechanistic modelling (QMM), a type of local model based on linking toxic effects to physicochemical properties for a specific case rather than a global QSAR model encompassing a number of different reactivity domains and MIEs. These QMMs bring a transparent mechanistic basis to modelling, and a number have been developed since for different mechanistic categories leading to skin sensitization.[17] These models, based on the interpretation of mechanistic chemistry, can be thought of as the precursor to the MIE.

The AOP framework was first outlined by Ankley *et al* in 2010.[18] Ankley presented the AOP as a conceptual framework, containing key information outlining the links between an MIE and an Adverse Outcome (AO) at a high level of biological organization relevant to risk assessment. Generalized examples of Key Events (KEs) along an AOP were presented by Ankley, including receptor/ligand interactions, DNA binding and protein oxidation. This conceptual framework is shown in Figure 1.

A number of case examples from ecotoxicology were also presented, including pathways for narcosis, photo-activated toxicity, the aryl-hydrocarbon receptor, activation of the estrogen receptor and impaired vitellogenesis. It was noted that a complete AOP with full elucidation of all steps is not necessary for the AOP to be a useful tool. Gaps in an AOP can be filled using weight-of-evidence or statistical approaches to establish links between exposure and adverse outcomes. Much effort can be saved by specifically targeting areas of the AOP designated as important for the assessment of a specific endpoint. The AOP framework for risk assessment complies with the NRC's vision for the future of toxicity testing, allowing for the identification of specific endpoints of regulatory concern and providing understanding of the toxicity mechanisms that cause them.

This initial framework was extended by Villeneuve and Garcia-Reyero early in 2011,[19] who identified the importance of predictive methodology in the application of AOPs. MIEs are critical interactions that can be modelled to develop (Q)SARs for predicting the likelihood of a chemical interacting with a specific target. In an analogous manner the AOP highlights key toxicity pathway events that can be linked to *in vitro* tests, and so can be used to predict or test a pathway. These enable the AOP framework to build upon previous risk assessment approaches, as well as to bring new science to bear on the problems and challenges faced by toxicology.

The European funded initiative SEURAT-1[20] started in January 2011, with the aim of developing a replacement for repeat-dose, systemic-toxicity *in vivo* tests. The strategy was to combine the use of *in vitro* and *in silico* methodologies, through a number of linked projects, to deliver results applicable in both the pharmaceutical, and consumer goods/cosmetics spheres. Of the seven projects, the most relevant to MIEs are COSMOS[21] and ToxBank[22]. COSMOS is focused on the development of *in silico* open access tools for the prediction of systemic toxicity endpoints for cosmetic products. The ToxBank project is developing a web-based warehouse for systemic toxicity data, a database and repository for test compounds, and tissue and cell banks for *in vitro* tests.

A first approach at linking the MIE to chemistry came from Enoch *et al* in 2011.[23] Enoch related the MIE to chemical category formation, using the fact that genotoxic chemicals are often electrophilically reactive, leading to the covalent modification of DNA. A number of mechanistic categories were developed to describe such MIEs, including acylation, Michael addition, Schiff base formation, S_N1, S_N2, and S_NAr. For each category, mechanistic and structural alerts were developed, combined into models know as *in silico* profilers, then assessed and discussed through a number of examples. Profilers such as these have been used to construct predictive

tools such as the OECD QSAR Toolbox.[24] Enoch noted that, while the predictions made using these methods were only as good as the toxicological data that was sourced to build them, chemical categories are useful because they are mechanistically driven, and help to provide an understanding of why a chemical is able to undergo a specific interaction. This work aligns well with the MIE, as it too is mechanistically driven. This approach builds upon earlier work linking electrophilic reactivity to human health endpoints and brings it into an MIE perspective. Originally, an electrophilic index was used to predict skin sensitization potential quantitatively using Read-Across QSAR techniques.[25] This was followed by the formation of electrophilic chemical groups for low molecular weight chemical compounds known to cause respiratory sensitization,[26] and these rules were later developed to link respiratory sensitization mechanisms to the electrophilic index.[27] These studies were published before the introduction of the MIE and AOP framework, but fit well with the ideas presented since, and conform to similar ideals.

A key step in defining the AOP, and terms used within it, came from the OECD in 2012, [28] when it collected relevant definitions of important terms from the AOP and toxicity pathway research frameworks. The OECD defined the AOP as a linear sequence of events from the exposure of an individual to a chemical substance through to an understanding of the adverse (toxic) effect at the individual level (for human health) or population level (for ecotoxicological endpoints). An AOP consists of a number of KEs that are intermediate between the MIE and an apical adverse outcome. These KEs must be toxicologically relevant to the apical outcome and experimentally quantifiable. A number of similar, but distinctly different, definitions for the MIE were presented by the OECD, including:

- The initial point of chemical-biological interaction within the organism that starts the pathway.[29]

- Direct interaction of a chemical with specific biomolecules.[19]
- The molecular level, chemical-induced perturbation of a biological system.
- Chemical interaction at a molecular target leading to a particular adverse outcome.
- The seminal interaction (e.g. DNA-binding, protein oxidation, or receptor/ligand interaction) of a chemical with a biological target.

The OECD recognized that the development and elucidation of AOPs will require contributions of multiple scientific fields, covering chemistry, biology and toxicology. Standardization of terms used in AOPs is necessary to allow these fields to communicate and develop the framework into the future.

Developments in 2013

In March 2013, the 7th amendment to the cosmetics directive 76/7678/EEC,[30] and subsequent cosmetics regulation 1223/2009[31] came into effect in Europe, enacting a ban on the use of animal testing for cosmetic ingredients. At this time, the availability of alternatives to *in vivo* methods appropriate for cosmetic product risk assessment varied greatly by endpoint. The commission noted that good progress had been made in the area of *in vitro* alternatives to measure basal cytotoxicity.[32] In other areas, such as eye irritation and skin sensitization, more work is required to provide adequate alternatives. This important milestone, in the transition from toxicology dominated by *in vivo* methods to one accepting the non-animal alternatives of *in vitro*, *in silico* and *in chemico*, provides a key industrial incentive to the development of these alternatives, including the MIE and AOP.

In a study analogous to an MIE approach to carcinogenicity, Benigni *et al* reviewed and combined a number of 2D SARs.[33] Carcinogenicity is broken down by mechanism of action to

yield structural alerts for receptor binding, oxidative stress, hormonal imbalance, and direct and indirect alkylation of DNA. Of nine structural alert classes across genotoxic and non-genotoxic carcinogens, a positive predictivity of greater than 70% was obtained in six cases. This computational methodology shows the promise of the integration of theoretical knowledge with *in vitro* assay information. Benigni notes that the addition of further structural alerts will allow for an expansion of this approach, increasing its impact.

Casalegno and Sello published a similar study predicting mode of action of environmental toxicants by structural similarity.[34] These models were based not on experimental data, but on structural features alone. This approach relies on the fact that the interaction between well-characterized molecules and an ill-defined biological target depends on the chemistry of both parts. Casalegno and Sello note that a lack of understanding of the modes of action associated with ecotoxicology makes this a difficult study, as poorly defined modes of action, such as narcosis or electrophilicity, are often used. Both a better understanding of the complex mechanisms behind ecotoxicity and a greater availability of data are required to make an approach such as this practically useful.

Gutsell and Russell's 2013 analysis, [35] shows that chemistry is key to understanding the MIE and also has other important roles to play in the AOP framework. Linking a defined dose of a chemical compound to an adverse effect requires the use of both chemical information, including analytical experiments, and theoretical techniques, such as (Q)SARs. An understanding of the chemical attributes that are required to generate an MIE can be used to filter the number of pathways that need to be considered in risk assessment. The MIE can be boiled down to a chemical interaction, and so a series of *in chemico* experiments coupled with *in silico* models

will be able to make risk assessment, based on chemistry alone, a reality. A visualization of this wet/dry cycle is shown in Figure 2.

AOP approaches were reviewed in mid-2013 by Vinken.[36] This review covered the development of AOPs from key building blocks, and several well-developed examples were discussed, including skin sensitization, cholestasis, liver fibrosis and liver steatosis. Vinken also discussed the key roles of (Q)SARs and *in vitro* tests to predict and to inform pathways. Key challenges in the field are presented, including compliance with the complexity of toxicology, the inclusion of dose-response relationships in pathway development, the integration of exposure and toxicokinetic data, and the transparent and objective evaluation of the outcomes.

Martin *et al* presented the use of mode of action to categorize aquatic toxicants and build models.[37] This study involved modes of action including receptor agonism, enzyme inhibition, chemical reactivity and biosystem disturbance. Martin notes that while the models provide promising results (overall prediction accuracy 85%), further categorization of the more general modes of action, such as chemical reactivity, into smaller structural-mechanism groupings (as may be expected with a more MIE-like analysis) should improve predictivity.

A key issue, particularly in ecotoxicology, is the extrapolation of data points between species during chemical risk assessment. This also applies to KEs within the AOP – including the MIE. LaLone *et al* described a quantitative tool for the extrapolation of MIEs between species using bioinformatics approaches and an understanding of the target species biology.[38] The conservation of KEs across several AOPs is an important simplification of biology within the AOP approach. If some of these KEs can also be preserved across species, this will aid the difficult task of ecotoxicological risk assessment, due to the diverse nature of the environment, and provide

additional data points in human toxicology, where results can be read across from existing animal studies.

An entirely quantitative risk assessment using the AOP framework was attempted by Maxwell *et al* at the end of 2013.[39] The skin sensitization AOP was one of the first AOPs to be considered well developed within the field.[28] Maxwell used KEs from early in the pathway to provide input parameters for the "total haptenated protein" model, with the "CD8+ T cell response" model providing predictions for KEs late in the pathway and for the overall adverse outcome (allergic contact dermatitis).

Chemical dose applied to the skin is linked to the amount of total haptenated protein using mathematical models, taking into account the pharmacokinetic steps as the chemical travels from skin surface to the protein. These include clearance mechanisms, metabolism, and other chemical transformations. The CD8+ T cell response model aims to predict the number of hapten-specific human CD8+ central memory T cells generated following repeated exposure to a chemical sensitizer, using inputs from the total haptenated protein model. Maxwell suggests that, once a threshold of hapten-specific human CD8+ central memory T cells is exceeded, an inflammatory response will manifest upon re-exposure to a sensitizer. These models were at the time in the early stages of development. However, by benchmarking them against clinical data it should be possible to predict whether a specific skin exposure will cause the required hapten-specific T cell response required to cause an adverse effect upon re-exposure.

In November 2013, Wu *et al* published a decision tree to predict Developmental And Reproductive Toxicity (DART) endpoints.[40] This *in silico* assessment was made without use of an AOP framework and shows the complexity associated with predicting across multiple

endpoints and toxicity pathways. Development of the decision tree identified a number of important structural fragments that can enhance the understanding behind DART modes of action using MIEs. The decision tree itself allows for rapid screening of potential DART.

Developments in 2014

In January 2014, Caldwell *et al* published an integrated approach for prioritizing pharmaceutical ingredients for ecotoxicological risk assessment using an AOP-informed approach.[41] Mammalian pharmacology data and pharmaceutical usage data were combined to provide a prioritization for risk assessment and advanced research. Prioritization of assessment is particularly important in ecotoxicology where the amount of data available to construct predictive models is often limited.

In 2014 the AOP Wiki was released as version 1.0.[42] This project represents a joint effort by the European Commission - Joint Research Centre and the U.S. Environmental Protection Agency, and serves as part of the OECD-sponsored AOP Knowledgebase. The AOP Wiki resource is designed to allow toxicology scientists to share AOP-related knowledge in an appropriate format in an open source forum. It also encourages the evaluation and acceptance of this research by the AOP community. The AOP Wiki represents an important tool in data-sharing and peer-review. Making research available through this wiki should be a priority for scientists working on AOPs and MIEs.

Computationally linking the reactivity of a chemical compound to its skin sensitization potential has been a key goal of the AOP community as the skin sensitization AOP is one of the best developed within the AOP Wiki. The MIE for skin sensitization is the covalent modification of a skin protein by a toxicant. Building on their earlier work,[15–17] Roberts and Aptula linked previously developed QMMs to local lymph node assay results for S_NAr electrophiles in 2014.[43]

This resulted in the development of a linear relationship between reactivity parameters and EC3 values measured experimentally. This analysis provides further evidence for the use of chemical reactivity as a descriptor for the analysis of the skin sensitization potential of novel molecules in the AOP framework.

Modern approaches to toxicology risk assessment must attempt to improve on existing *in vivo* methods. Leist *et al* highlighted this in 2014, pointing out that limited resources and high cost associated with animal methods have led to a large number of chemicals being untested, and mixtures are rarely evaluated at all.[44] Leist proposed a new vision, running chemicals and mixtures through *in silico* and *in vitro* approaches, using toxicity pathways, mode of action and AOP frameworks to gain a comprehensive evaluation. Only if no conclusive results can be obtained would animal testing be carried out. An understanding of the MIEs of these chemicals will aid in the generation of a comprehensive risk assessment of many chemicals without the use of *in vivo* experiments. A large number of under-tested chemicals and mixtures could be risk-assessed using this high-throughput method, as illustrated in Figure 3.

Molecular modelling is one area of *in silico* science that shows much promise in its ability to study the MIE. One such approach was published by Tsakovska *et al* in 2014.[45] A model was developed for the PPARγ receptor as a target of interest for liver steatosis. The receptor binding pocket was analyzed using PPARγ complexes with full agonists from the Protein Data Bank (PDB),[46] and a pharmacophore was developed encompassing the most important features for binding and their role in PPARγ activation. As may be expected for a receptor binding MIE, the pharmacophore consists of hydrogen bonding, hydrophobic and aromatic interactions between the ligand and PPARγ. Models such as this provide *in silico* screens to identify potential steatogenic inducers early in risk assessment.

Following their previous work into reactivity-driven MIEs for DNA modification,[23] Nelms *et al* used a similar approach to develop a collection of structural alerts built into an *in silico* profiler for systemic toxicity of hair dyes.[47] Repeat dose toxicity studies for 94 hair dyes were used, and chemicals were grouped based on structural similarity. Four categories were identified for hair dyes containing a 2-nitroaminobenzene, a 4-nitroaminobenzene, an aromatic azo, or an anthraquinone moiety. Nelms went on to develop a mechanistic hypothesis for each of the four groups, and refined structural alerts were presented as an *in silico* profiler, covering pro-quinones, quinones, meta-substituted benzenes and aromatic azo compounds. This profiler assigned 56 of the 94 chemicals in the dataset to a mechanism-based chemical category. The reactivity-driven nature of these toxicity mechanisms may also allow the alerts to be used in mitochondrial toxicity, and this was also discussed by Nelms. This paper emphasizes that approaches like this do not attempt to predict oral repeat-dose toxicity, but instead a particular MIE that might be responsible for an AOP leading to chronic toxicity. Detailed studies such as this are required to develop models for MIEs, and to identify the MIEs themselves, as at this time they are generally poorly understood at a mechanistic level.

The workshop "Advancing AOPs for Integrated Toxicology and Regulatory Applications" took place in 2014. One report from this workshop by Tollefsen *et al* mentions the pivotal role of MIEs.[48] MIEs measured through *in silico*, *in vitro* and *in chemico* techniques were discussed and shown to have an important part to play in the AOP framework. MIEs cover a wide variety of chemical interactions, and these were highlighted through examples, including receptor binding MIEs leading to endocrine disruption and reproductive toxicity, as well as covalent binding to skin proteins leading to skin sensitization and allergic contact dermatitis, as has been discussed

above.[28] These discussions highlight the significance of the MIE and the important roles different areas of toxicology will play in gaining an understanding of these key interactions.

Chemistry's key role in MIE and AOP research was further highlighted by Allen *et al.*[49] AOP networks for a number of well-understood toxicants were developed using literature searches. These case studies were used to unify existing definitions of the MIE and concluded that an MIE is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome *via* a pathway. The role of the MIE in AOP research and toxicity risk assessment was also discussed, including a framework in which an *in silico* (Q)SAR relates molecular properties of a novel compound to an MIE, and any associated AOP can then infer an expected adverse outcome for the compound, as shown in Figure 4. By establishing an understanding of the chemistry behind interactions between molecules and biomolecules or biosystems (Q)SARs can be constructed, allowing the MIE to directly contribute to toxicity screening processes and, later, with further quantitative understanding, risk assessments.

Madden *et al* published a report on the development of AOPs in ecotoxicology in late 2014,[50] noting that AOPs can receive criticism due to their simplistic nature which makes them poor reflections of complex toxicological processes. In response, they point out that an AOP should never be seen as a complete picture, but as a flexible tool that will improve over time as new data is constantly added. Even if an AOP is incomplete, it can still provide a large amount of useful information in a risk assessment. *In vitro* to *in vivo* extrapolation (IVIVE) of doses also presents a challenge due to the difference between *in vitro* experiments and *in vivo* situations. For example in an *in vitro* assay does not consider the metabolism of a chemical. Effective integration of data from *in chemico*, *in silico*, *in vitro* and *in vivo* will be required to overcome this, and the AOP Wiki,[42] among other resources,[22,24,51,52] provides a way to achieve this.

As has already been discussed,[23,33,47] structural alerts play a key part in predictive toxicology. Chemotypes were introduced in 2014 as a new way of coding such alerts, and Chemotyper is software that allows them to be searched against a dataset.[53] Chemical substructure mark-up language (CSRML), an XML-based code, is used to capture chemotypes. This language allows for the inclusion of physico-chemical properties and descriptors as well as 2D structure, allowing greater flexibility of constructed models.

Developments in 2015

In February 2015, a report from FutureTox II (held in January 2014) was published by Knudsen *et al*[54] The goals of this meeting were to discuss the progress towards the NRC's vision for toxicity testing in the 21st century[1] and *in silico* and *in vitro* methodologies featured heavily. AOPs were discussed as a key concept. Despite OECD guidelines which state that an AOP links a single MIE and an effect at a high level of biological organization, it is accepted that a single MIE may lead to several AOPs and an adverse effect may be associated with several MIEs. AOP networks can capture the complexity of the biological sphere far better; and the MIE is identified as an important KE that can be used to screen compounds to identify the AOPs that are likely to be of most interest.

The difficulty associated with IVIVE has already been mentioned.[50] Angrish *et al* presented an approach to bridging the IVIVE gap in high throughput screening (HTS) assays.[55] Using an ultra-sensitive gas-phase probe molecule, they aim to measure effects on metabolism that will link through to understood *in vivo* outcomes. As an example, the pharmacokinetic parameters associated with a cytochrome-2A6-driven metabolism were identified in order to measure the effect of a toxicant indirectly by observing a metabolite: methyl tertiary-butyl ether.

Methodology such as this allows MIEs and other KEs to be directly linked to relevant *in vivo* data.

Steinmetz *et al* utilized open-source data to construct a screening tool for retinoic acid receptor binding using the MIE principle.[56] 2D fragments of binders were coded in SMARTS based on "rules" derived from the PDB. In addition to this, physicochemical properties (vertex adjacency information magnitude,[57] number of rotational bonds, molecular weight and logarithm of the water-octanol partition coefficient) were used to give an insight into the physico-chemical applicability domain for retinoic acid receptor binding. These early approaches towards MIE tools are designed not to replace *in vitro* testing or be a complete *in silico* model, but rather to provide a rapid screen and prioritization methodology to assist in risk assessment.

The importance and potential impact of AOPs as a framework in non-animal risk assessment was reviewed by Burden *et al* in March 2015.[58] Key to the recent paradigm shift in toxicity testing is the Replacement, Refinement and Reduction of animal methods - also known as the 3Rs.[59] The AOP framework provides an important scientific basis on which new risk assessment practices can be built - as no single alternative testing method will replace an *in vivo* model like-for-like. One such combined approach could involve the prediction of adverse effects based on a pre-determined MIE. Concerted efforts to collect, integrate and organize data from relevant sources across scientific disciplines will be required to reap the benefits of such a coherent framework. Regulatory input is also required, to guide the use of AOPs in risk assessment decision-making.

While single AOPs are considered the building blocks of AOP development, they are not a complete representation of complex biological systems when considered in isolation. AOP networks would seem to provide a solution to this, as described by Knapen *et al*. [60] AOPs are

constructed from MIEs, KEs and AOs that can be shared across multiple pathways. Where these common elements are shared, AOPs can be combined into AOP networks. This principle is demonstrated in the case of DART, where aryl hydrocarbon receptor, estrogen receptor, aromatase and androgen receptor-based MIEs lead to pathways that converge in DART endpoints. This AOP network is shown in Figure 5. The analysis of networks such as this conveys a number of advantages over considering AOPs in isolation. Not only are AOPs in isolation unrealistic, by combining them into networks of assays, measurement of a single MIE or KE can provide information on a number of different endpoints and pathways, improving testing efficiency. The more understanding that can be gained about the MIEs, KEs, and their networks the more potential combinations will come to light, further benefitting risk assessment.

Judging the quality of AOPs will be an important step in their development to become a staple of the chemical risk assessment procedure. This is discussed by Becker *et al* who present a number of examples using the OECD approved Bradford-Hill considerations for the assessment of confidence in an AOP.[61] These considerations include the biological plausibility and empirical evidence (i.e. dose-response) for KE relationships (KERs), and the essentiality of KEs (i.e. are downstream events prevented if an upstream event is blocked?). Each consideration is broken down, with the aim being to assign a high, moderate or low confidence in each KE or KER. Several case studies are given and the importance of using weight-of-evidence-based approaches to analyze AOPs discussed. To ensure transparency and promote consistency across AOP research, these kinds of confidence assessments must be carried out and analyzed. This ultimately will lead to enhanced rigor, transparency and reproducibility for AOP confidence assessment, improving confidence in AOPs themselves.

This particular area of AOP research has been further explored by Yauk *et al*[62] The AOP for alkylation of DNA in male premeiotic germ cells leading to heritable mutations was examined in depth in order to establish its biological plausibility and the empirical evidence that supports it. In turn each KE was considered, examining how the KE works and how it was measured or detected experimentally. KERs are then examined, looking at how the biological processes work, the weight of evidence that supports them, any uncertainties or inconsistencies in their supporting data, and the level of quantitative understanding of the linkage they provide. Following this analysis the KEs and KERs are assessed as having strong, moderate, or low levels of confidence. A particular challenge, noted by Yauk, in this AOP development was the gathering of appropriate dose-response data for assessment of the Bradford-Hill considerations from historical studies that were not conducted with this type of research in mind and which are time-consuming to interpret. The AOP framework should be able to overcome this hurdle, since the more KEs and KERs are isolated, the more likely it is that new AOPs will incorporate KEs and KERs which have already been characterized, and so the process will speed up.

Dent *et al* discussed the AOP for anti-androgenic activity in humans in a similar level of detail.[63] The aim was to establish the status of the tools and approaches being put towards a non-animal risk assessment for this AOP. A particular point noted here was the high level of uncertainty associated with the extrapolation of data from effects seen in high dose animal studies to the much lower exposures anticipated for humans. Therefore, the exposure of the human to the chemical will be key. If human exposure is not significant, is a detailed risk assessment necessary at all?

Risk assessments for metals pose a number of different issues compared to those for organic chemicals. Von Stackelberg *et al* used an AOP approach to analyze the neurodegeneration

endpoint caused by the exposure of humans to metals and mixtures of metals, specifically lead, arsenic, cadmium and manganese.[64] An AOP was developed and assessed using the Bradford-Hill considerations. This AOP linked the activation of extracellular-signal-related kinase by metals to an increase in levels of cellular calcium ions and neurological disorders including deficits in learning and cognition. It is important that MIE and AOP approaches can be utilized in these different situations to increase their impact in toxicology.

Following on from previous work into *in silico* models for reactivity driven MIEs for DNA modification[23] and skin sensitisation[47], Nelms *et al* published the development of an *in silico* profiler for analyzing mitochondrial toxicity in June 2015.[65] Category formation based on structural similarity was performed on a set of pharmaceutical drugs with mitochondrial toxicity data. Once categories had been decided, a literature search was undertaken to elucidate mechanistic information behind the MIE, and other KEs in AOPs associated with mitochondrial disruption. Structural alerts were defined and coded in SMARTS using information from the literature and substructures from the category formation. The structural alerts highlight molecules that exhibit toxicity as protonophores, redox cyclers, and inhibitors of the complexes of the electron transport chain. With a small amount of data available, this study has been able to gain insight into several key MIEs for mitochondrial dysfunction, and provide a profiler that can be utilized to screen large data sets to identify chemicals with the potential to induce mitochondrial toxicity.

A number of AOPs leading to hepatic toxicity were compiled by Mellor *et al* in late 2015.[66] This work links the agonism and/or antagonism of several nuclear receptors to hepatic steatosis *via* a number of converging pathways. These pathways are currently reasonably well understood from

a biological standpoint, but further research is required to elucidate key information about the MIE and build structural alerts or other models to be able to predict this endpoint.

Difficulty in understanding MIEs can be aided by finding new ways to measure them, and this was demonstrated by Sanderson *et al* using NMR spectroscopy.[67] The binding of molecules to protein residues features in MIEs for skin sensitization and hepatotoxicity among others. The rate of this reaction could be used to inform toxicological risk assessments quantitatively, providing an *in chemico* link between molecule and extent of protein modification. NMR spectroscopy was used to measure these rates for the reactions of electrophilic organic chemicals (representing different mechanistic classes) and simple amines and thiols (representing lysine and cysteine protein side chains respectively). *In chemico* assays, such as this, will be important in informing MIE and AOP driven toxicological risk assessment, providing vital quantitative information and experimental validation.

Constant development of new AOPs has led to the discovery and characterization of new MIEs, for example, the chemicals binding to tubulin identified by Marchetti *et al* in late 2015.[68] This MIE leads to aneuploidy offspring – a teratogenic disorder causing an abnormal number of chromosomes. The majority of data for the MIE and KEs of this AOP come from rodent studies. They are thought to be conserved in humans, allowing the extrapolation of the existing *in vivo* data for use in human toxicology. Similarities in the mechanism of action across several phyla and the high homology between mouse and human tubulin provide evidence for this. This MIE presents an interesting case, in which *in silico*, *in chemico*, *in vitro* and *in vivo* studies may all be able to be used to predict the impact of an MIE in humans.

The main drive in the development of AOPs up to this point has been for the risk assessment of cosmetic products and industrial chemicals. AOPs can also find use in drug development, as was discussed by Patlewicz and Fitzpatrick in December 2015.[69] They focused mainly on the use of *in silico* tools, particularly (Q)SARs, for the prediction of KEs along an AOP. While a number of tools, some of which we have already discussed, have been developed previously, the number of KEs requiring prediction will continue to demand more from (Q)SAR scientists. It is particularly highlighted that AOPs must be kept in mind during the development of new (Q)SARs, promoting the development of tools which model small steps between KEs and not large leaps over many complex levels of biological organization.

At the end of 2015 the SEURAT-1 initiative closed and the EU-ToxRisk program,[70] was initiated early in 2016 to continue driving mechanism-based toxicity testing for risk assessment in the 21st century.

CONCLUSIONS

As toxicology moves away from animal experiments and towards *in silico*, *in vitro* and *in chemico* methods, as well as combinations of these, deeper understanding and new tools are required. The AOP framework provides a powerful method to do this. As arguably the most important key event within the AOP, a greater knowledge of MIEs will be vital to the success of this framework. A greater understanding of the MIE must be developed, and tools must be constructed to allow MIEs to be predicted, and measured for novel compounds.

The MIE sits on the boundary between biology and chemistry, and as such both these fields have important roles to play in its development. This has been shown in this review, with a number of

publications coming from both a chemical and biological background. *In silico*, *in vitro* and *in chemico* methods all have a part to play, and this is also represented.

As the first KE in an AOP, the MIE has a key role to play in the understanding of toxicity in both humans and animals. As a result the MIE is applicable in both human health and ecotoxicology risk assessment. The MIE and AOP provide a template for the coming together of these fields, as it is expected that some, but not all, of the KEs and KERs will be shared across species. One of the main drives of the AOP framework is for better risk assessment procedures, and this collaboration can help by providing additional data and understanding previously confined to their fields.

As outlined above a number of MIEs have now been characterized. This allows us to begin grouping these interactions based on their chemistry. The earliest studied MIEs were covalent bond forming reactions between chemicals and biological molecules. These MIEs include the reactions of electrophilic chemicals with DNA molecules,[23] and the covalent modification of skin proteins.[43] MIEs such as these can lead to toxicological endpoints such as allergic contact dermatitis,[39] genotoxicity, and immunological disorders.[39]

A second category of MIEs is those which involve non-covalent binding of a chemical to a biological target such as a receptor or enzyme. These can include the activation of PPARγ,[45] or the agonism or antagonism of nuclear receptors,[66] both leading to liver steatosis, the inhibition of complexes of the mitochondrial electron transport chain leading to mitochondrial dysfunction,[65] and the binding of chemicals to tubulin leading to teratogenic endpoints.[68]

A final category of MIEs is for those chemicals that do not directly interact with a specific biomolecule, but rather cause a disturbance in cellular or organelle biosystems. Biosystem

disturbance has been alluded to in several cases[37,49] and provides an MIE category for the narcosis mode of action often used in ecotoxicology which is associated with membrane disruption.[34] Other biosystem disturbance MIEs include chemicals which act as protonophores or redox cyclers leading to mitochondrial toxicity endpoints.[65]

The impact of the MIE and AOP framework has undoubtedly increased over the last three years, with the number of publications contributing to the area increasing each year. The understanding gained, and tools developed so far, represent an important platform for future development, with the ultimate aim being a tool for quantitatively predicting the impact of a chemical on a human or ecotoxicological target. The emergence of the MIE highlights the idea that chemical understanding is critical in modelling, and that mechanistic local modelling is key to providing the best predictions. Feeding an MIE prediction tool into a combined AOP framework, incorporating exposure, absorption, distribution, metabolism and excretion (ADME), and an understanding of downstream effects along the AOP will be able to provide a genuine alternative for toxicology: reliable risk assessment entirely free of *in vivo* experiments.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Data Statement

According to the University of Cambridge data management policy, all the data used in this paper is available either in the paper or in the SI. A copy of the data is also available in the University of Cambridge repository at: https://www.repository.cam.ac.uk/

ABBREVIATIONS

AHR, aryl hydrocarbon receptor; AOP, adverse outcome pathway; CRSML, chemical substructure mark-up language; DART, developmental and reproductive toxicity; E2, estradiol ; GtH, gonadotrope hormone; HTS, high throughput screening; IVIVE, *in vivo in vitro* extrapolation; KE, key event; KER, key event relationship; MIE, molecular initiating event; NRC, national research council; PDB, protein data bank; QMM, quantitative mechanistic model; (Q)SAR, (quantitative) structure activity relationship; T, testosterone; VTG, vitellogenin.

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Dr Timothy E. H. Allen is a post-doctoral research associate in the group of Professor Jonathan Goodman at the Department of Chemistry, University of Cambridge having completed his Ph.D. in Chemistry in the same group in 2016. His research interests include studying the MIE from a chemical perspective, identifying the chemical characteristics that allow such interactions to occur and constructing computational models based on them.

Professor Jonathan M. Goodman did experiments on aldol reactions during his PhD with Ian Paterson at the University of Cambridge, and then was a post-doc with Clark Still at Columbia University, before returning to Cambridge, where he is now Professor of Chemistry and is investigating how knowledge of chemistry and molecular structures can contribute to issues in toxicology.

Dr Steve Gutsell obtained his PhD in Organic Chemistry from the University of Wales, Swansea and is currently a Computational Chemist at Unilever's Safety and Environmental Assurance Centre. His research is concerned with using predictive methods such as (Quantitative) Structure-Activity Relationships ((Q)SAR), Read Across and other techniques to predict both toxicological and ecotoxicological activities from chemical structure. Recent areas of interest

include how pathways-based approaches can be used to create novel risk assessments for consumer products.

Dr Paul Russell obtained his PhD in Pharmaceutical Science from King's College London and is currently Chemistry Strategic Science Leader at Unilever's Safety and Environmental Assurance Centre. His research interests are the development of non-animal tools and approaches to define toxicity pathways from Molecular Initiating Events in support of the safety assessment of chemicals in consumer goods.

FIGURES

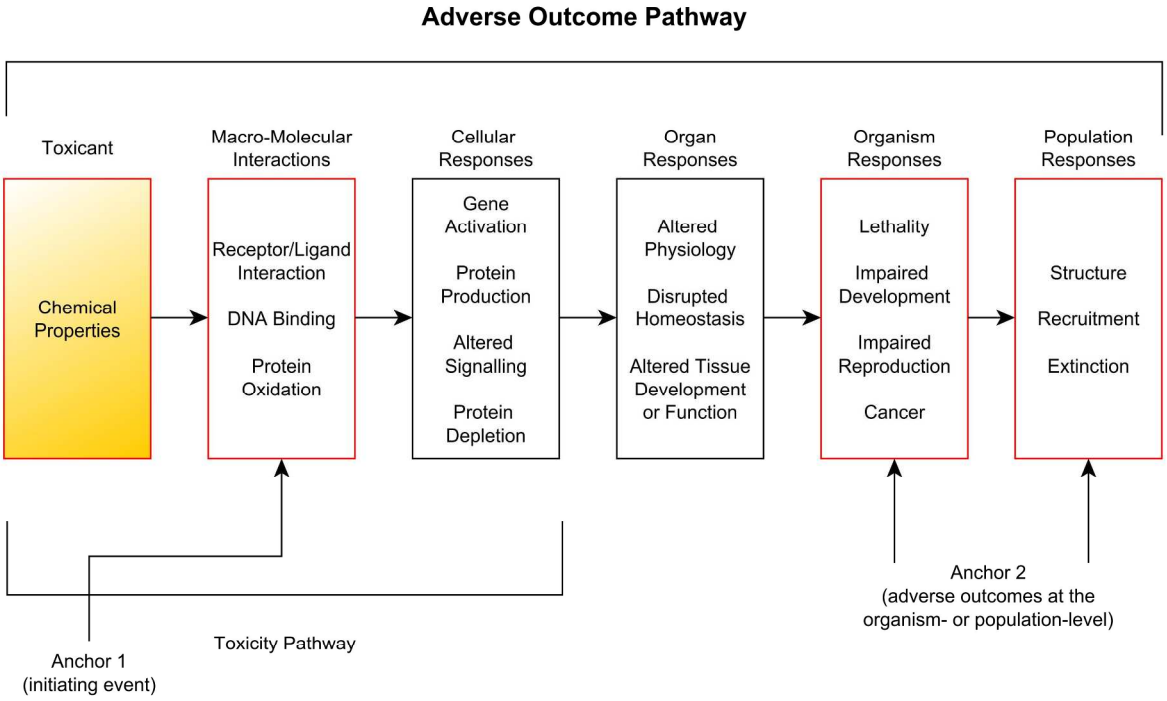

Figure 1. Ankley's conceptual diagram of an AOP, including the MIE (Anchor 1). Adapted with permission from Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrrano, J. A., Tietge, J. E., and Villeneuve, D. L. (2010) Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem., Vol. 29*, 730–741,[18] from John Wiley & Sons Inc.

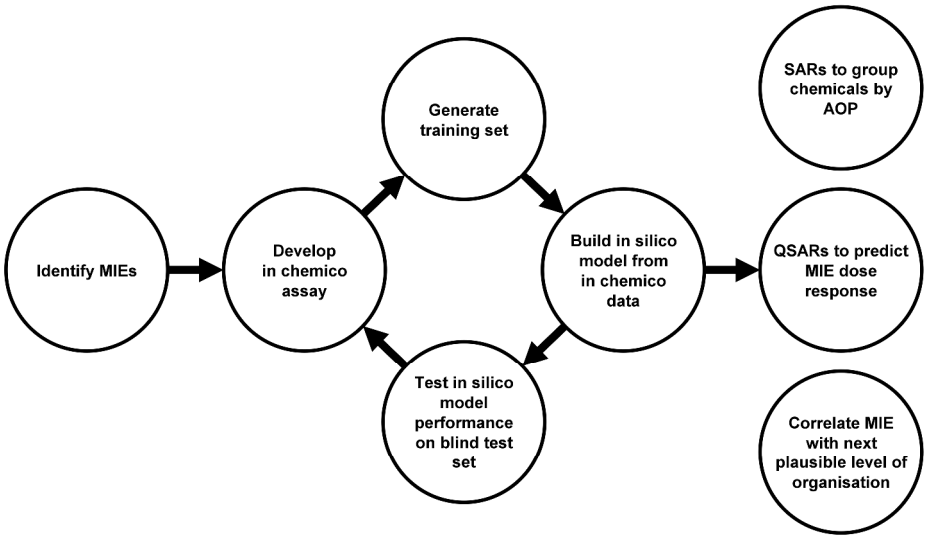

Figure 2. A wet/dry cycle for the development of in silico models. Adapted from Gutsell, S., and Russell, P. (2013) The role of chemistry in developing understanding of adverse outcome pathways and their application in risk assessment. *Toxicol. Res., Vol. 2*, 299–307,[35] with permission from The Royal Society of Chemistry.

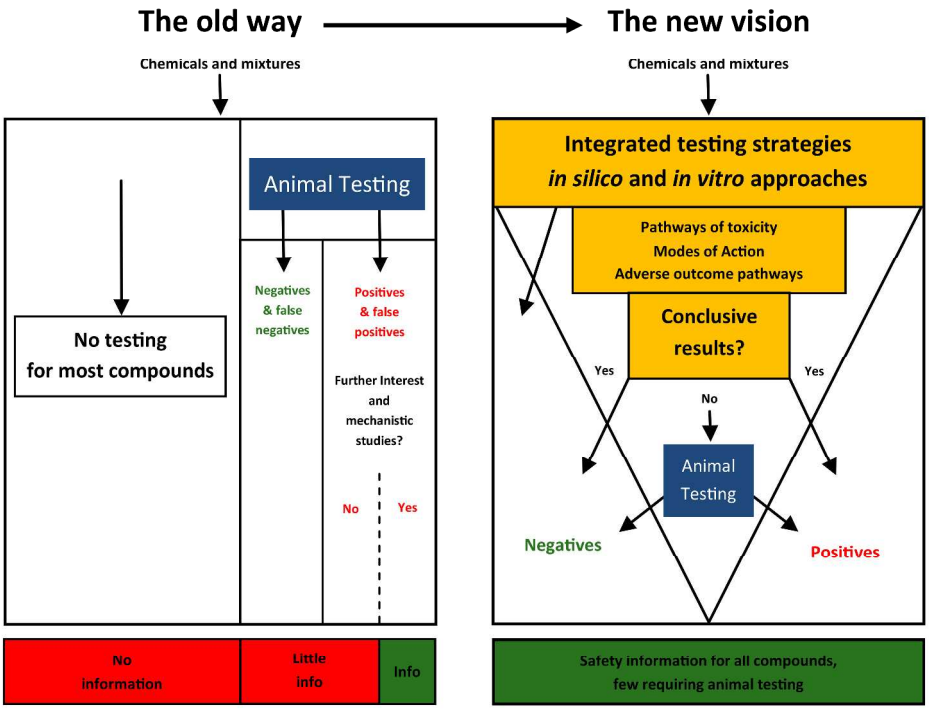

Figure 3. Leist's new vision for the future of toxicity testing. Adapted with permission from Leist, M., Hasiwa, N., Rovida, C., Daneshian, M., Basketter, D., Kimber, I., Clewell, H., Gocht, T., Goldberg, A., Busquet, F., Rossi, A. M., Schwarz, M., Stephens, M., Taalman, R., Knudsen, T. B., McKim, J., Harris, G., Pamies, D., and Hartung, T. (2014) Consensus report on the future of animal-free systemic toxicity testing. *ALTEX 31*, 341–356.[44]

Figure 4. Framework for a (Q)SAR approach based around MIEs. The (Q)SAR relates molecular characteristics to the MIE and the AOP infers an adverse outcome from the MIE. Adapted from Allen, T. E. H., Goodman, J. M., Gutsell, S., and Russell, P. J. Defining Molecular Initiating Events in the Adverse Outcome Pathway framework for risk assessment. *Chem. Res. Toxicol. 27*, 2100–2112. Copyright 2014 American Chemical Society.[49]

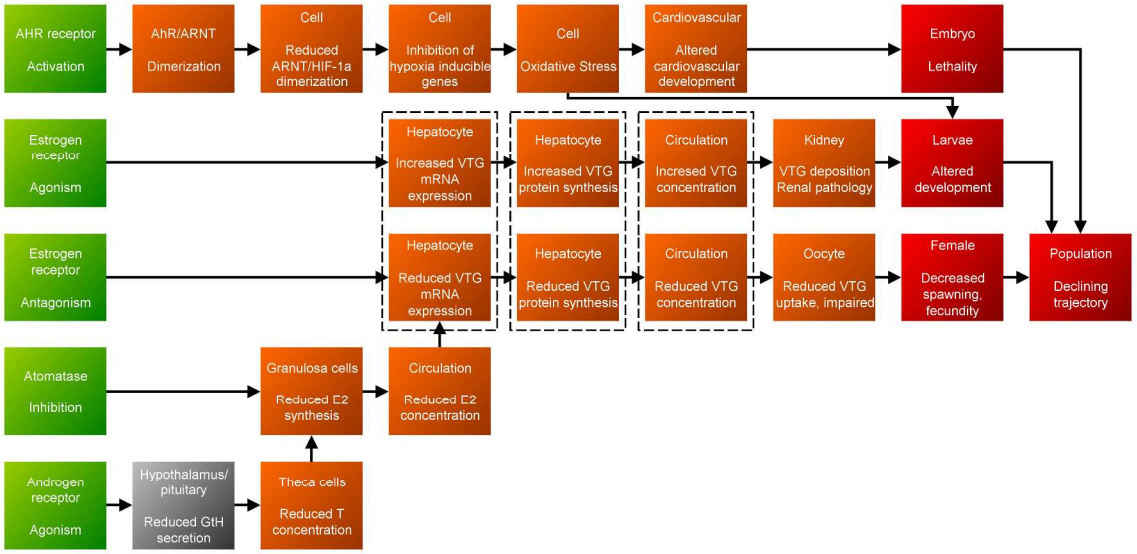

Figure 5. An example of an AOP network based on five reproductive and developmental toxicity-related AOPs in fish available on the AOP wiki. MIEs are indicated in green, KEs in orange and adverse outcomes in red, as per the AOP wiki template. The grey box represents a KE with a "weak" weight of evidence. The dotted squares indicate KEs that are defined as changes in the opposite direction of the same biological component. AHR: aryl hydrocarbon receptor, E2: estradiol, GtH: gonadotrope hormone, T: testosterone, VTG: vitellogenin. Reprinted from *Reproductive Toxicology*, Vol. 56, Dries Knapen, Lucia Vergauwen, Daniel L. Villeneuve, Gerald T. Ankley; The potential AOP networks for reproductive and developmental toxicity assay development; pp 52-55; Copyright 2015;[60] with permission from Elsevier.